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Contribution of vitamin D and calcium-sensing receptors methylation to the risk of colorectal cancer in Saudi patients

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Abstract

Aim: Colorectal cancer (CRC) is one of the most common cancers worldwide. It results from an interaction between genetic and epigenetic alterations with micronutrients. Vitamin D, via the vitamin D receptor (VDR) and calcium sensing receptor (CaSR), stimulates several hallmarks of cancer. This study aimed to measure the methylation status of VDR and CaSR in CRC patients and correlate them with other clinicopathological parameters to identify their role as diagnostic biomarkers.

Material and Methods: The study was performed on 108 participants (CRC patients and controls). RT-PCR was used to measure the expression of VDR and CaSR mRNAs, whereas pyrosequencing was used to identify the methylation status of the promoter using DNA samples.

Results: The expression results showed that neither VDR expression nor CaSR expression had a significant correlation with CRC risk. However, the promoters of VDR and CaSR were highly hypomethylated in CRC patients (the fold change was -7.09 for VDR and -4 for CaSR).

Discussion: Although VDR and CaSR had a strong correlation with cancers, the results showed that they might not be promising diagnostic markers for CRC. However, more experiments on larger sample size are needed to elucidate the correlation between promoter methylation modification and CRC carcinogenesis.

Keywords

Colorectal Cancer, VDR Promoter Methylation, CaSR Promoter Methylation

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Introduction

In the past decades, colorectal cancer (CRC) has been the least common type of cancer and could be described as rare. It has become the third leading cause of death related to cancers in developed countries [1]. In Saudi Arabia, CRC is considered the second most common type of cancer, ranked first and third among the male and female populations, respectively [2]. There are a number of risk factors for which CRC needs more research [3]. Diet firmly impacts the danger of CRC, and changes in nourishment propensities may diminish the occurrence of this malignant growth burden [4]. The efficacy of calcium and vitamin D as chemopreventive agents against CRC is supported by strong biological plausibility in human studies [5,6]. These affect several hallmarks of cancer, such as promoting differentiation, adhesion, proliferation, inflammation, and the cell cycle, as well as inhibiting oxidative DNA damage and modulating the cell signaling pathways associated with CRC [6]. Although the effects of these nutrients may be mediated by polymorphisms of the vitamin D receptor (VDR) and calcium sensing receptor (CaSR), this binding enables the transactivation of the target genes involved in cellular differentiation, apoptosis, angiogenesis, proliferation, calcium homeostasis, and cellular growth kinetics [6].

DNA methyltransferases (DNMTs) add the methyl group to the 5' position of cytosine to produce 5-methyl cytosine [7]. Generally, in non-promoter regions, the tumor suppressor and DNA repair genes are mostly hypomethylated, whereas in promoter regions, they are mostly hypermethylated. Moreover, gene silencing, genomic instability, apoptosis, DNA repair, and cell cycle control are strongly affected by hypermethylation in cancers including CRC [7].

Since previous studies have shown that VDR and CaSR can act as chemopreventive agents against CRC, they can be used as molecular biomarkers or as potential targets for CRC prevention. From this point of view, this study aimed to measure the expression levels of VDR and CaSR mRNA in the blood samples of CRC patients. The methylation of the promoter regions of both the CaSR and VDR was also assessed.

Material and Methods

Study design

The research committee of the Biomedical Ethics Unit at the Faculty of Medicine, King Abdulaziz University, approved this study (reference no. 379-17). This study was performed on 46 controls and 62 CRC patients and was conducted at King Fahd Medical Research Center (Cancer and Mutagenesis Unit) from July 2021 to May 2022. Samples for whole blood were drawn into lavender-top vacutainers containing EDTA from each participant. All CRC participants were selected according to the following criteria: 1) Saudis of any age, 2) who agreed to participate and provide a blood sample to be used in DNA and RNA extractions, 3) were diagnosed with CRC at any clinical stages, and 4) with fully documented histopathological and treatment profiles of CRC patients. For controls, the inclusion criteria included 1) Saudis of any age, 2) who agreed to participate and provide a blood sample to be used in DNA and RNA extractions, and 3) who were not diagnosed with any metabolic syndromes or having any family history of cancer and not undergoing any treatment during the study period.

Synthesis of cDNA by reverse transcriptase and RNase inhibitor treatment

RNA was extracted from whole blood samples using a QIAamp RNA Blood Mini Kit (QIAGEN, 52304). The final concentration of RNA was determined at 260 nm using a Nanodrop DeNovix DS-11 spectrophotometer. All RNA samples were stored at -80°C. The cDNA was prepared from separated RNA samples (300 ng) using the random primer scheme of a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, 4368814]. RNase inhibitor treatment (Thermo Fisher Scientific, N8080119) was used to inhibit RNase activity. The thermocycler reactions were programmed following the manufacturer's protocol. All cDNA samples were stored at -20°C.

Real-time polymerase chain reaction (RT-PCR)

Primer sequences of VDR and CaSR were obtained using the USCS browser. The mRNA sequences were obtained, and using the Primer3 web tool, primers were designed after confirming their characteristics. The following set of primers were used in the RT-PCR reaction: VDR [forward primer, 5'-GACACACTCCCAGCTTCTCT-3', Tm = 60.5°C, and reverse primer, 5'-GCTCTAGGGTCACAGAAGGG-3', Tm = 62.5°C]; CaSR [forward primer, 5'-TCAAATCAAGGCCGGAGTCT-3', Tm = 58.4°C and reverse primer = 5'-GCTGGGCTGCTGTTTATCTC-3', Tm = 60.5°C]. Finally, GAPDH primers were as follows: forward primer, 5'-CACATCGCTCAGACACCATG-3', Tm = 60.5°C, and reverse primer 5'- ACCAGAGTTAAAAGCAGCCC-3', Tm = 58.4°C. To perform RT-PCR, 20 µl/well of the SYBR® Green Master Mix reaction was designed following the instructions of the Thermo Fisher Scientific (A25741) kit. For each gene, three independent experiments were performed.

Determination of methylation status using real-time polymerase chain reaction (RT-PCR)

DNA samples were extracted from the whole blood using a QIAamp DNA Mini Kit (Qiagen, 51306). DNA concentration was measured by the DeNovix DS-11 spectrophotometer at 260 nm. The final DNA samples were stored at -20˚C. The EpiTect Bisulfite Kit (QIAGEN, 59104) was used for complete bisulfite conversion and DNA cleanup for methylation analysis. The PCR primer sequence for VDR, CaSR, and COL2A1 was obtained using the USCS browser. To perform RT-PCR, 20 µl/well was prepared using 2x EpiTect MethyLight Master Mix, primer and probe solutions, RNase-free water, and converted DNA (Table 1). The RT-PCR was done using real-time cyclers from Applied Biosystems.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 7. Descriptive data were expressed as the mean ± standard error of the mean (SEM). The comparison of physical parameters between the two groups was performed using an unpaired t -test. The expressions of VDR and CaSR were normalized to the expression of GAPDH using the REST 2009 software. The normalized results were then compared using an unpaired t-test with Welch's correction to calculate the degree of differences between the two independent groups. A one-way ANOVA test with Bartlett's test was used to determine whether there were any statistically significant differences between the means of VDR and CaSR expression and dietary intake.

A p<0.05 was considered statistically significant. Regarding methylation experiments, each sample was performed in duplicate for greater accuracy. The mean was then calculated and the relative expression of VDR or CaSR was calculated using the Livak method.

Table 1. Real-time PCR (RT-PCR) primers used for the detection of promoter methylation

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
VDR	GTTGTTTTTGTTTGTTAAAAGGC Tm = 55.7°C	GACGAATAAACAAACTATTCCG Tm = 56.6°C
CaSR	GGTTTTTTCGTATAGTTTC Tm = 60.9°C	AACCGTAACATAAAAAAC Tm = 56.7°C
COL1A2	TCTAACAATTATAAACTCCAACCAA Tm = 62.8°C	GGGAAGATGGGATAGAAGGGAATAT Tm = 64.2°C
	Probes (5'-3')	
VDR	TAGCGGAGTCGTGTGCGTCGGGAGC Tm = 74.0°C	
CaSR	TTTCGGTCGTGGGTTTTTACGAGGATGAGT Tm = 70.7°C	
COL1A2	CCTTCATTCTAACCCAATACCTATCCCACCTCTAAA Tm = 73.2°C	

Where, VDR: vitamin D receptor; CaSR: calcium- sensing receptor; COL2A1: collagen type II alpha 1 chain.

Table 2. Physical characteristics comparison between patient and control groups.

Variable	Patients N= 62	Controls N= 46	p value
Age (year)	56.52 ± 1.54	52.13 ± 1.83	0.07
Height (cm)	165.1 ± 1.18	164.5 ± 1.17	0.71
Weight (kg)	73.76 ± 2.00	83.78 ± 2.58	0.0024**
Waist (cm)	101.1 ± 2.54	104.0 ± 2.83	0.45
Hip (cm)	110.5 ± 2.39	106.5 ± 2.79	0.29
WHR	0.92 ± 0.02	0.99 ± 0.02	0.02*
BMI (kg/m2)	27.08 ± 0.73	31.04 ± 0.98	0.0012**

Variables are presented as mean ± SEM; BMI: Body Mass Index; WHR: Waist to Hip Ratio; *p<0.05; **p≤0.001

Table 3. Relationship between (VDR and CaSR) mRNA expressions in CRC patients and healthy controls with gender.

Gene	Gender	Mean ± SEM, (n)	p value
VDR	Female Controls	28.30 ± 0.65, (n=18)	0.81
	Female CRC	28.05 ± 0.86, (n=15)	
	Male Controls	26.72 ± 0.46 (n=28)	0.01*
	Male CRC	28.58 ± 0.50, (n=47)	
	Female Controls	28.30 ± 0.65, (n=18)	0.04*
	Male Controls	26.72 ± 0.46 (n=28)	
	Female CRC	28.05 ± 0.86, (n=15)	0.59
	Male CRC	28.58 ± 0.50, (n=47)	
CaSR	Female Controls	32.58 ± 1.78, (n=17)	0.33
	Female CRC	34.65 ± 0.74, (n=14)	
	Male Controls	34.94 ± 0.55, (n=26)	0.38
	Male CRC	35.78 ± 0.65, (n=44)	
	Female Controls	32.58 ± 1.78, (n=17)	0.15
	Male Controls	34.94 ± 0.55, (n=26)	
	Female CRC	34.65 ± 0.74, (n=14)	0.36
	Male CRC	35.78 ± 0.65, (n=44)	

*p<0.05 (Unpaired t test)

Results

Demographic and environmental analyses of the study participants

The CRC patients (n = 62) were divided into two groups according to their gender—males (n = 47, 75.81%) and females (n = 15, 24.19%). Several physical characteristics were studied, as shown in Table 2. In comparing the physical characteristics between the patients and the controls, the unpaired t-test results showed significant differences between the patient and control groups in weight (p = 0.0024), body mass index (BMI) (p = 0.0012) and waist to hip ration (WHR) (p =0.02).

Regarding the effect of environmental factors on CRC progression, three factors (smoking, family history of cancer, and nutrition) were analyzed in this study. Smoking was an ineffective factor in this sample of CRC patients (n = 62) as well as in healthy controls (n = 46). Only 6.45% (n = 4) of CRC patients were currently smoking, while 61.29% (n = 38) were non-smokers, and the remaining 32.26% (n = 20) were ex-smokers. The proportion of healthy control smokers was 17.39% (n = 8), non-smokers 67.39% (n = 31), and ex-smokers 15.22% (n = 7). Regarding the cancer family history, only 3.22% (n = 2) of 62 CRC patients had family history of CRC, 20.97% (n = 13) of patients had family history of cancers other than CRC, such as breast, gastric, prostate, brain, uterus, leukemia, lymphoma, liver, and lung cancers, while the rest of CRC patients (75.81%, n = 47) did not have any family history of cancer. On the other hand, in the (n = 46) healthy subjects, none of the control subjects had a family history of CRC or any other types of cancer. Finally, regarding the nutritional factor, especially the intake of calcium from natural sources such as milk yogurt, nuts, eggs, and cheese, patients, and controls were categorized into four groups based on their intake: no, low (monthly intake), moderate weekly intake), and high (daily intake(. The percentage of CRC patients who did not receive calcium from any diet or supplementation sources was 11.29% (n = 7), followed by 20.97% (n = 13) who consumed low calcium products ,35.48% (n = 22) who consumed moderate calcium products weekly, and 32.26% (n = 20) who consumed products with high calcium levels daily. On the other hand, the percentage of healthy controls who consumed calcium sources monthly in their diet was 30.43% (n = 14), weekly 26.09% (n = 12), and the remaining 43.48% (n = 20) of the controls took calcium daily.

Expression of VDR and CaSR mRNA

The expression level of VDR in CRC patients' blood samples was 0.63-fold higher than in controls, with a mean of 28.45 ± 0.43 (n = 62) vs. 27.34 ± 0.39 (n = 46), and p = 0.07. The CaSR was expressed with a 0.57-fold change higher in CRC patients compared to controls, with a mean of 35.71 ± 0.50 (n = 62) vs. 34.01 ± 0.79 (n = 46), and p = 0.06.

Relationship between VDR and CaSR mRNA expressions in CRC patients and healthy controls with gender

The CRC patients and controls were divided according to their gender into two groups: female patients (n = 15, 24.19%) and male patients (n = 47, 75.81%). The controls were divided into female controls (n = 18, 39.13%) and male controls (n = 28, 60.87%). The unpaired t-test comparison (Table 3) showed that there was no significant difference in the expression of CaSR mRNA between the female category either in the CRC patients

group or the controls as well as between the male patients and the male controls (p = 0.33 and 0.38, respectively). On the other hand, VDR mRNA expression showed a significant difference between male CRC patients and male healthy controls (p = 0.01) as well as between male and female controls (p = 0.04) but was not significant in the female category (CRC vs. controls) (p = 0.81).

Genetic and environmental interactions for the CRC patients' group

The genetic and environmental factors, smoking and the intake of calcium from natural sources were determined to assess their contributions to CRC tumorigenesis. Regarding smoking, CRC patients were classified into two groups—non-smokers and ex-smokers (n = 58, 93.55%) versus current smokers (n = 4, 6.45%). The expression difference of VDR mRNA between smoker and non-smoker groups was not significantly different (p = 0.76) [mean of VDR was 28.96 ± 1.056 (n = 4) vs. 28.42 ± 0.45 (n = 58) in smokers and non-smokers, respectively]. For CaSR mRNA expression, an unpaired t-test also showed a non-significant difference (p = 0.69) between the two groups [smokers 36.34 ± 0.76 (n = 4) vs. non-smokers 35.51 ± 0.52 (n = 58)]. Regarding calcium source intake, CRC patients were categorized into three groups: low, moderate, and high intake. Expression of VDR and CaSR mRNA was measured in the low consumption group versus the moderate and high calcium sources consumption group. The expression of VDR between the three groups of CRC patients showed a non-significant difference (p = 0.84). In contrast, the CaSR mRNA expression showed a very high significant difference between the patient group according to calcium sources intake (p≤0.0001).

Determination of the promoter methylation status of VDR and CaSR genes

The mean of methylated VDR gene promoter (ΔCT) was (40.08) in controls compared to (35.67) in CRC patients. The comparison between gene expression (ΔΔCT) of VDR with COL1A2 expression showed that the gene expression difference level was approximately 7.35 times higher in the controls, whereas it was reduced to 0.26 in CRC patients. The fold change level (2-ΔΔCT) of VDR methylation expression between CRC patients and controls was -7.09, which indicates that this gene promoter region is highly hypomethylated in CRC blood samples compared to healthy blood samples. This level could be expressed as a 136.24 difference change. Regarding CaSR promoter methylation status, the mean of methylated CaSR gene promoter (ΔCT) was 38.72 in controls compared to 37.57 in CRC patients. The comparison between gene expression (ΔΔCT) of CaSR with COL1A2 expression showed that the gene expression difference level was approximately 6 times higher in the controls, whereas it was reduced to 2 times in CRC patients. The fold change level (2-ΔΔCT) of CaSR methylation expression between CRC patients and controls was -4, which indicates that this gene promoter region is hypomethylated in CRC blood samples compared to healthy blood samples. This level could be expressed as a (16) difference change.

Discussion

CRC is one of the most prevalent cancers in Saudi Arabia, as the number of deaths from CRC is alarmingly increasing [2]. Patients diagnosed with localized CRC have a much better 5-year survival rate compared to patients diagnosed with metastasized CRC; therefore, early screening of risk factors for CRC is vital [3]. DNA methylation is associated with promoter regulatory regions of almost all housekeeping genes, as well as with half of tissue-specific genes. Promoter hypermethylation of the tumor suppressor gene has been associated with decreased gene transcription [7].

Therefore, there is an urgent need to understand the molecular mechanisms of CRC and develop effective biomarkers that have a critical role in the prevention of CRC. Researchers had to understand the complex genetic interactions of certain micronutrients, proteins, signaling pathways and their relation to the growth and development of CRC [5,6]. Vitamin D and calcium functions are mediated by special receptors (VDR and CaSR). Vitamin D metabolism, with the help of VDR and CaSR, stimulates several hallmarks of cancer. Hence, VDR and CaSR are genes that could have an important role in CRC tumorigenesis [6].

The possible association of risk of CRC with vitamin D, VDR polymorphisms, and CaSR polymorphisms has been discussed; however, the results were controversial. VDR gene was first identified in colon cancer cells in 1982 [8]. VDRs are expressed in normal and malignant colorectal tissues and in a wide range of tissues [9]. It has previously been reported that VDR expression is different in normal human tissues and colon tumor samples. Studies have shown down-regulation of VDR expression in colon tumor samples [10-12]. CaSR is crucial for the maintenance of extracellular calcium homeostasis by affecting parathyroid hormone secretion and calcium reabsorption [13]. CaSR has been implicated in breast and prostate cancers [14]. CaSR expression was found to be weak or absent in colon carcinomas and was inversely correlated with differentiation status [15]. In the current study, the expression levels of VDR and CaSR in the blood of CRC patients were determined to assess their contributions to CRC carcinogenesis. The results revealed that none of the two receptors was related to CRC pathogenesis, as the expression of both of them was not significantly different in the blood of CRC patients compared to healthy controls. The upregulation of CaSR expression is influenced by 1,25(OH)2D3 in colon cancer cells [16].

CaSR expression positively correlates with differentiation and apoptosis markers but negatively correlates with proliferation markers in samples from human colorectal adenocarcinomas [17]. A large prospective study on CRC patients suggests that increased tumor CaSR expression is an independent predictor of CRC-specific mortality [18].

In conclusion, in the current study, expression levels of VDR and CaSR were measured in the blood of CRC patients. However, the results showed a non-significant difference. This research needs to be performed on a larger number of CRC patients as well as on colorectal cancerous tissues with further improvement and development that pointed out preclinical and epidemiological parameters.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and

approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
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